CHANGES IN LIPID ORGANIZATION OF UTERINE EPITHELIAL CELL MEMBRANES AT IMPLANTATION IN THE RABBIT

E. Winterhager and H.-W. Denker

Institut für Anatomie der RWTH Aachen
Melatener Strasse 211
D-5100 Aachen, West Germany

INTRODUCTION

Interaction of the blastocyst with the endometrium at implantation starts with adhesion of the apical membrane of the trophoblast to the apical membranes of the uterine epithelium (Enders and Schlafke, 1972; Schlafke and Enders, 1979). In the rabbit this event is followed by apical membrane fusion of both partner tissues thus leading to the formation of a mixed symplasm, and by invasion into the endometrium (Enders and Schlafke, 1971). At the same time, widespread fusion of the lateral cell membranes of the uterine epithelial cells starts. This uterine epithelial cell fusion occurs in two overlapping phases, in the antimesometrial portion of the endometrium from 7 days post coitum (7 d p.c.) associated with attachment and invasion of the abembryonic trophoblast (yolk sac placenta), and mesometrially on the endometrium of the placental folds from 8 d p.c. (associated with chorioallantoic placenta). Uterine symplasms formed in this way are found exclusively in the implantation chamber, i.e., in the vicinity of a blastocyst, and are very large, containing hundreds of nuclei.

Fusion of the lateral (formation of endometrial symplasms) as well as the apical membranes (mixed symplasm of trophoblast plus uterine epithelium) could depend on alterations in membrane composition leading to changes in membrane fluidity. In general, membrane fusion is accompanied by an increase in membrane fluidity which is often thought to be its prerequisite, as reported by Chandler (1984) for exocytosis and by Wolf et al. (1981) for fertilization. Anionic phospholipids have been proposed to be involved in increasing membrane fluidity so favoring membrane fusion (Papahadjopoulos, 1978). On the other hand cholesterol is known to increase membrane rigidity (Shinitzky, 1984).

For rabbit uterine epithelium, the concept of a functional interrelationship between the two types of lipids is tested here using freeze-fracture histochemistry with probes for both lipids that may permit detection of membrane microdomains. The peptide antibiotic polymyxin B (PXB), has been reported to bind specifically to anionic phospholipids in membranes (Hartmann et al., 1978; Sixel and Galla, 1981). Complexes of polymyxin B with anionic phospholipids can be detected as characteristic lesions on freeze-fracture replicas. For the localization of cholesterol filipin was used as a probe. Filipin interacts specifically with 3-β-hydroxy sterols (Elias et al., 1978; 1979) and leads to well defined membrane protrusions which can be detected on freeze-fracture replicas as well as in ultrathin sections.

Present address: Institut für Anatomie, Universitätsklinikum, Hufelandstr. 55, D-4300 Essen 1, West Germany
Interest was focused on possible differences in lipid composition of the uterine epithelial membranes in the implantation chamber where fusion processes are extremely widespread, or in the blastocyst-free segments as well as in the uterine epithelium of pseudopregnant animals where lateral cell fusion is limited (Busch et al., 1981; Winterhager et al., 1984).

MATERIALS AND METHODS

Sexually mature female rabbits (mixed breeds) were caged individually on a 12 hour light/12 hour dark cycle and were fed a standardized pellet diet. Pseudopregnancy was induced by injection of 75 I.U. human chorionic gonadotropin (hCG) i.v. (Prolan®, Bayer, Leverkusen, FRG). Mating was performed with two bucks of proven fertility. The day of mating and day of hCG injection were designated as day 0. Material for transmission electron microscopy was obtained from pseudopregnant (6, 7 days post hCG injection, p.hCG) as well as pregnant (6, 7 d p.c.) animals. In the case of pregnancy the endometrium of the implantation chamber and the blastocyst-free segments were investigated separately. Freeze-fracture histochemistry was exclusively performed on day 7 p.c./p.hCG.

Polymyxin B Treatment

Rabbits were anaesthetized by sodium pentobarbitone (Nembutal®, 0.4 ml/kg body weight i.v.), the uterus was exposed and polymyxin B (Upjohn Co.) was applied by injection of a 1% to 7% solution in physiological saline into the uterine cavity. Before injection the uteri were ligated into short segments (about 1 cm). Care was taken not to impair blood supply. After different times of incubation (1, 2, 5, 10, 20, and 30 minutes) uteri were fixed by vascular perfusion as described previously (Winterhager and Kühnel, 1982).

Filipin Treatment

Filipin (Sigma) was solubilized in dimethylsulfoxide (DMSO) (1 mg filipin/50 µl DMSO) (= filipin stock solution). Incubation with filipin was performed with pieces of endometrium that had been immersion fixed (2.5% glutaraldehyde in 0.1 M cacodylate buffer for 4 - 6 hours). After opening the uterine cavity, uterine segments were treated with filipin at different concentrations of the stock solution (0.05% and 0.02%) and different incubation times (1, 2, 4, 6, 8, and 12 hours) at room temperature in the dark. Controls were treated with DMSO alone.

Transmission Electron Microscopy Ultrathin Sections And Freeze-Fracture

Specimens from perfusion-fixed uteri were routinely embedded in araldite. The PXB and filipin-treated endometria as well as controls, were processed for freeze-fracturing after several washings with cacodylate buffer as described previously (Winterhager and Kühnel, 1982). Replicas were produced in a Bioetch apparatus 2005 (Leybold Heraeus). Replicas and ultrathin sections were examined in an EM 10 (Zeiss) electron microscope.
RESULTS

Morphology

At the time of trophoblast invasion, the apical membrane of uterine epithelial cells undergoes dramatic changes in its configuration in the implantation chamber. The microvilli which are thin and short in the preimplantation phase transform into numerous swollen and irregularly shaped cell projections in the implantation chamber beneath the adjacent trophoblast prior to and during attachment (Figure 1). These cell projections seem to collapse and fuse with one another forming ring-like structures. The deformation of the microvilli into cell organelle-free projections is accompanied by an umbrella-like distension of the apical cell region (Figure 1), thereby overlapping the neighboring cell like a tile. As a consequence, the lateral membranes are tilted in the apical region and this part which contains the broad tight junctional belt may now run nearly parallel to the cell apex.

In the crypts, these blunt projections of the apical plasma membrane probably fuse with those of opposite cells (Figure 2). This process appears to play a role in the formation of the exceedingly large uterine epithelial symplasms which proceed to cover virtually the whole antimesometrial surface of the endometrium, smoothing out many former depressions.

Distribution Of Cholesterol

Pseudopregnancy And Blastocyst-Free Segments In Pregnancy

Cholesterol complexes are detected in the plasma membrane at 7 d.p.hCG/p.c. mainly in the form of typical lesions, i.e., circular protuberances of approximately 20 - 25 nm in diameter on the P-face and depressions on the E-face (Figure 3, inset). In the uterine epithelial cells of pseudopregnant rabbits as well as of the blastocyst-free segments of pregnant does, these lesions seem to be homogeneously distributed in the apical as well as lateral membranes. Apical membranes exhibit more filipin-cholesterol complexes than the lateral membranes (compare Figure 3 with Figure 4). On the lateral membranes, however, the number of lesions decreases from the apical to the basal portion of the membrane (Figure 4). This fact does not seem to be due to hampered diffusion of the reagent into the tissues as indicated by endothelial cells of underlying capillaries which are highly decorated with lesions. In contrast to the observations on the reaction with PXB (see below), the pattern of filipin lesions is homogeneous on all epithelial cells throughout the whole length of the uterus. Microdomains on the lateral or apical membranes, i.e., regions with specialized reaction patterns, are restricted to the membrane region of the tight junctional belt. Within this area a reaction with filipin is not detectable. A small strip of membrane below the belt shows a reduced number of lesions.
Pregnancy: Implantation Chamber

Most lateral membranes of the uterine epithelium in the implantation chamber exhibit many more cholesterol-filipin complexes than those of blastocyst-free segments of the same stage (7 d) of pseudopregnancy. Alternatively, a few lateral membranes can be found which are nearly free of cholesterol-filipin complexes. In that case the complexes which are seen, appear in small clusters (Figure 5). All apical membranes are decorated extensively so that the membrane does not show any smooth area (Figures 5 and 6). This pattern is found in the apical membranes of the whole epithelium surrounding the blastocyst.

In one respect, however, the localization of membrane cholesterol-filipin complexes in the implantation chamber is similar to that in the blastocyst-free segments and in pseudopregnancy: a gradient of lesions, i.e., a number that decreases towards the basal region, is seen on the lateral membrane in all cases.

The tight junctional area is again nearly free, the numerous gap junctions which are exclusively found between the epithelial cells of the implantation chamber at this reproductive stage (Winterhager et al., 1988) are totally free of filipin-cholesterol protrusions (Figure 7). Even in membrane areas where gap junctions seem to be formed (formation plaques, indicated by loosely aggregating particles) no reaction with filipin is obtained.

Figure 1. Uterine epithelium of the implantation chamber (7 d p.c.) with confronting trophoblast (T). The microvilli of the uterine epithelium are transformed into irregularly shaped cell projections forming partly ring-like structures (arrow). The cell apex is distended, shifting thereby the upper parts of the lateral membranes into a nearly surface-parallel position. X5,300

Figure 2. Apical cell surfaces of uterine epithelial cells in the implantation chamber (7 d p.c.) located vis-à-vis in the crypts. The nearly organelle-free, ectoplasmic cell projections interdigitate, obliterating the lumen. X10,000

Figures 3 - 4. Freeze fracture cytochemistry with filipin as a probe for cholesterol, pseudopregnancy.

Figure 3. Freeze fracture replica of uterine epithelium of a pseudopregnant animal (7 d p. hCG) incubated with filipin (0.2%, 3 hours). The apical membranes are decorated with typical protrusions whereas the region of the tight junctional belt is devoid of such reaction products. X24,500. The arrowhead indicates the direction of shadowing in all freeze fracture micrographs. Inset: Higher magnification of filipin/cholesterol complexes reveals circular protrusions (20-25 nm) on the P-face and mainly depressions on the E-face. X65,000

Figure 4. Lower portion of the lateral cell membranes of the same cell as in Figure 3. Membrane lesions are less frequent than in the apical membrane and their number decreases towards the basal portion. X24,500
Figures 5 - 7. Freeze fracture immunocytochemistry with filipin as a probe for cholesterol: pregnancy. Uterine epithelial cells of the implantation chamber (7 days p.c.); incubation with 0.2% filipin for 4 hours.

Figure 5. Labeling with filipin is enhanced on the apical membranes (a) as well as on most lateral membranes (see right side of the figure). Some lateral membranes, however, exhibit only few lesions arranged in clusters (lower left). X25,200

Figure 6. The apical membranes of the uterine epithelial cells in the implantation chamber (7 d p.c.) exhibit abundant circular protrusions indicative for cholesterol. X20,600

Figure 7. The membrane area with numerous gap junctional proteins is free of filipin/cholesterol complexes. X57,600
Distribution Of Anionic Phospholipids

Pseudopregnancy And Blastocyst-Free Segments In Pregnancy

Uterine epithelial cell membranes of pseudopregnant rabbits and those of the blastocyst-free segments of pregnant rabbits show the same reaction pattern of negatively charged phospholipids with PXB, at implantation time (7 d p.hCG/p.c.). Figures 8a, b, c demonstrate different types of membrane lesions that can be observed. This polymorphism includes an aggregation of particles combined with circular particle-free protrusions on the P-face (Figure 8a) or elongated protrusions on the P-face associated with intramembranous particles (IMPs) which seem to be associated with depressions of similar shape on the E-face (Figure 8b). With increasing incubation time membranes are more often seen to decompose and seem to form vesicles (Figure 8c). Results are evaluated using 7% PXB and an incubation time of 10 minutes. This methodological approach is chosen based upon an obvious balance between labeled, unlabeled, and destroyed cells. In addition to the polymorphism of lesions just described, the reaction to PXB is quite different between adjacent uterine epithelial cells. Cells without any reaction to PXB can be found that are located next to cells with perturbations of their membranes (Figure 9). However, the type of reaction with PXB is homogeneous within each single cell. Apical as well as lateral membranes of a given epithelial cell always exhibit the same kind of membrane lesions. The cell-to-cell differences in reaction pattern to PXB do not disappear after longer incubation times (up to 30 minutes). The number of cells which are destroyed by the detergent action of PXB, however, increases but even then unaltered cell membranes (i.e., without lesions) are detectable adjacent to those that have disintegrated into vesicles (Figure 9).

Except for the tight junctional membrane area (Figure 8a) (which is never decorated) compartments or microdomains on the lateral or apical membranes are not detectable.

Thus, a detailed evaluation of the results suggests that there are methodological problems that need to be discussed and which may result from the fact that treatment with PXB has been performed on native membrane material (see Discussion).

Pregnancy: Implantation Chamber

To obtain results with PXB on the epithelial membranes of uterine cells in the vicinity of the blastocyst, the concentration of PXB as well as the incubation time have to be considerably reduced, otherwise total cell destruction is observed. Concentrations between 1.5 to 3.5% and an incubation time between 1 and 2 minutes are found sufficient to get well defined lesions on the membranes. In contrast to the results obtained in pseudopregnancy and in the blastocyst-free segments (pregnancy) at day 7, the type of membrane lesions due to PXB is relatively homogeneous. These appear as particle aggregations or as elongated protrusions associated with the IMPs as illustrated for pseudopregnancy in Figure 8b. The reaction on the lateral as well as apical membranes is the same, whether cells are still non-fused or are already transformed into symplasms. Some of the lateral membranes, however, appear more damaged as indicated by a wavy structure and
segregation of proteins and lipids (Figure 10). Microdomains (except the tight junctional region, as already described) are not detectable on either membrane compartment. Gap junctions, which are frequently found do seem to be affected by PXB treatment. This means some gap junctions exhibit agglomerated junctional particles (Figure 10), and in other plaques, particles are dispersed.

**DISCUSSION**

Changes in the morphology of the apical plasma membrane of the uterine epithelium in relation to embryo implantation have been seen not only in the rabbit (as described briefly in the present communication) but also in a number of other species and may indeed be a general phenomenon (Leiser, 1979, 1980; Enders and Schlafke, 1967, 1972; Enders, 1975; Wooding et al., 1980; Murphy and Martin, 1985). Particularly obvious in the vicinity of the blastocyst, microvilli become transformed into blunt cell projections of considerably varying shape and size. This phenomenon is accompanied by flattening of the apical plasma membrane and tilting of the apical portion of the lateral membranes. The described morphological changes in the apical plasma membrane have to be discriminated from the well known dome-shaped cell protrusions related to maternal progesterone serum levels. Traditionally, such apical protrusions have been viewed as a sign of apocrine secretion (Beier and Kühnel, 1973) or as specializations for endocytosis in the rat (Enders and Nelson, 1973). In the rabbit, the apical protrusions are already considerably reduced in number in the implantation chamber when trophoblast attachment commences.

Figures 8 - 10. Freeze fracture cytochemistry with polymyxin B as a probe for anionic phospholipids.

Figures 8a, b, c. Different membrane reactions obtained with polymyxin B treatment (7% for 10 minutes) on uterine epithelial cells 7 d p. hCG (pseudopregnancy). a) Circular protrusions are similar to those obtained with filipin and are devoid of particles; the tight junctional area is free of reaction products, X33,000; b) Elongated protrusions with agglomerated particles, X50,000; c) Membranes decomposed into vesicle-like structures, X50,000.

Figure 9. Uterine epithelial cells of a blastocyst-free segment at pregnancy (7 d p.c.) incubated with 7% polymyxin B for 30 minutes. Two neighboring cells with different reaction to PXB: the membrane of the left cell is totally decomposed whereas the adjacent cell demonstrates a membrane without any reaction product. X15,800

Figure 10. Lateral membrane of a uterine epithelial cell of the implantation chamber (7 d p.c.) treated with 3.5% polymyxin for 1 minute. The membrane shows a wavy structure with segregated lipid areas (big arrow) and agglomerated gap junctional proteins (small arrow), whereas the tight junctional area with intercalated gap junctions does not appear affected. X31,700
All these phenomena suggest that the physico-chemical properties of the membranes and of underlying cytoskeletal elements may undergo alterations at this phase. Consequently it appears reasonable to ask whether the lipid composition of the apical membrane with respect to the phospholipid and/or cholesterol content has changed. This conclusion has already previously been drawn for the rat (Murphy and Martin, 1984, 1985; Murphy and Dwarte, 1987) using a similar methodological approach. This study likewise suggests that both types of lipids investigated, i.e., cholesterol and anionic phospholipids, undergo changes in density and distribution in the uterine epithelial membranes at implantation also in the rabbit.

However, these observations must be interpreted with caution since artefacts may be generated. The heterogenous reaction of the uterine epithelial cells to PXB (in pseudopregnancy as well as in the blastocyst-free segments in pregnancy) is an example. The non-reaction is surely not due to a total absence of anionic phospholipids. This current series of differing treatment times suggests that some of the anionic phospholipid molecules may indeed be masked, since prolonged incubation increases the amount of membranes with alterations. Severs and Robenek (1983) have suggested that PXB may be adsorbed by negatively charged glycoproteins, or that the anionic phospholipids may alternatively be masked by calcium. Murphy and Martin (1984) observed non-reactivity to PXB of the lateral epithelial cell membranes of the endometrium in the rat. They interpret these observations as evidence for differences in anionic lipid content but without excluding other unknown causes. Nevertheless, it appears to be of interest that this method does reveal differences in reactivity between membranes of uterine epithelial cells, of, on one hand, pseudopregnant animals and the blastocyst-free segments of pregnant animals, and, on the other hand, cells surrounding the blastocyst. The cells of pseudopregnant animals and the blastocyst-free segments are characterized by mosaic-like reactions to PXB whereas in the implantation chamber a homogeneous reaction of apical as well as lateral membranes indicates a more homogeneous composition.

In the implantation chamber, epithelial cells have previously been shown to be coupled by newly formed gap junctions (Winterhager et al., 1988). This must be expected to give rise to greater similarity due to metabolic cooperation. In addition to greater uniformity of reaction, the density of characteristic lesions in the implantation chamber probably indicates an increased concentration of anionic phospholipids and/or better accessibility. The latter could be due to a reduction of the thickness of the glyocalyx of the apical cell membrane particularly in the implantation chamber (Anderson and Hoffman, 1984).

The selectivity of filipin for binding to cholesterol is well established for simplified membrane model systems (Kinsky et al., 1967; Norman et al. 1972; Verkleij et al., 1973). However, it has been suggested that the complexity of cell membranes may in certain cases lead to false negative results due to a stabilizing influence of proteins (in the case of coated pits, the clathrin coat) (Feltkamp et al., 1982; Severs and Simons, 1983; Steer et al., 1984). Consistent with this stabilizing influence is the current finding that filipin plaques are absent in gap junctions of the uterine epithelium. The lower number of lesions in tight junctional membrane areas is difficult to interpret in this context since the role of proteins integral to or associated with the tight junctions is still incompletely understood (Citi et al.,
The observed increase in the number of plaques in the apical membrane in the implantation chamber, in contrast, cannot be explained on the basis of interference of proteins since in freeze fracture particle density is found to be increased (Winterhager, 1985) a phenomenon which also holds true for the rat (Murphy and Swift, 1983). If not due to changes in the glycolcalyx as discussed above for PBX-binding one would therefore assume that the apical plasma membranes of uterine epithelial cells show an increased content of cholesterol in the implantation chamber. Since diffusion problems cannot be excluded completely for the lateral membranes, it appears less certain whether the observed lower number of lesions does indeed show that the cholesterol content is less here than in the apical membranes. The same reservation may apply for the gradient seen in the lateral membrane with most lesions being formed in the apical portion.

It is remarkable that in the implantation chamber the filipin binding shows more diversity on the lateral membranes than in other parts whereas the reactivity of the apical cell membrane is more uniform.

With regard to implantation physiology, the reported results are in many respects unexpected for the distribution of anionic phospholipids as well as for cholesterol. Keeping in mind that many fusion processes are going on at the lateral membranes (uterine symplasm formation) as well as at the apical membranes (fusion implantation), it is expected to find a decrease in membrane cholesterol content and an increase in anionic lipids based on results obtained in simplified model systems (Yanagimachi et al., 1972; Bearer and Friend, 1981; 1982). Not withstanding the methodological problems discussed above, the current results would suggest that the concentration of anionic lipids increases - or they have better access to the probe - in the implantation chamber, combined with an increase in the cholesterol content, particularly in the apical membranes. About the same is found for the cholesterol content in the apical membranes of the uterine epithelium in the rat which seems to be increased at implantation stage as compared to a non-pregnant animal (Murphy and Dwarte, 1987). In the rat, however, fusion does not occur, neither for lateral membranes or between trophoblast and uterine epithelium. Alternatively, studies performed in other systems indicate that fusion can be independent of membrane rigidity and sterol content. Apical membranes of villous goblet cells, for example, exhibit abundant filipin-sterol complexes during secretion, i.e., more than nonsecretory cells (Trier and Madara, 1984). Conversely, enhanced membrane fluidity is not necessarily a prerequisite for cell fusion since photolabeling experiments show that virus-induced fusion in a variety of cell types is not accompanied by increased lateral mobility of cell membrane components (Aroeti and Harris, 1986).

In conclusion, the results presented on freeze fracture histochemistry using filipin and PXB as probes suggest that uterine epithelial plasma membranes do undergo changes in lipid (cholesterol and anionic phospholipids) composition in preparation for trophoblast attachment and implantation. In spite of the fact that some details need to be clarified due to certain problems inherent to the application of this methodology to complex tissues, the results suggest that the cholesterol content of the apical epithelial cell membranes increases at implantation in the vicinity of the blastocyst. This is in contrast to what one could expect when a simple fusion model as derived from liposome studies would be applied (White and Helenius, 1968; Papahadjopoulos, 1973) and also to findings obtained with sperm
(Bearer and Friend, 1981, 1982). Obviously the uterine epithelium/trophoblast system is very complex and components other than the two classes of lipids studied here may come into play, including e.g., integral membrane proteins and the cytoskeleton. Cell fusion related to embryo implantation is not a peculiarity of the rabbit. Fusion between uterine epithelial cells occurs in carnivores (Leiser, 1979; Enders, 1972), while fusion of trophoblast with uterine epithelium occurs in ungulates (Wooding et al., 1980) and a group of marsupials (peramelids, cf., Padykula, 1976). The processes of fusion that occur in the rabbit implantation chamber should provide a useful model system to study the cell biology of this phenomenon.

**SUMMARY**

The uterine epithelium of the rabbit shows conspicuous morphological changes of the cell membranes during the preimplantation and implantation phases, and finally undergoes cell fusion of both homologous (uterine epithelial cells with each other) and heterologous type (uterine epithelium with trophoblast). In the present investigations, it is asked whether these membrane alterations may be due to changes in lipid composition of the membranes.

Polymyxin B as well as filipin are used as histochemical probes to detect anionic phospholipids and cholesterol, respectively, in the membranes of the uterine epithelium at implantation. Typical lesions produced by these probes are visualized on freeze fracture replicas. The implantation chamber is being separately investigated and compared to the blastocyst-free segments and the endometrium of pseudopregnant animals of the same stage.

Filipin-induced lesions increase enormously in the apical and in most of the lateral membranes of the epithelial cells of the implantation chamber suggesting that the content of cholesterol is higher here than in the blastocyst-free segments and in pseudopregnant animals. The reaction with polymyxin B (anionic phospholipids) is found to be very heterogeneous with respect to the reaction products, in both the epithelium of pseudopregnant animals and the blastocyst-free segments. This heterogeneity disappears in the epithelium of the implantation chamber resulting in a homogeneous and strong reaction of apical and lateral membranes.

The results are discussed in the light of model experiments that have previously been done e.g., with liposomes, suggesting that negatively charged phospholipids increase membrane fluidity and that cholesterol is responsible for membrane rigidity. It is concluded that the described membrane alterations and fusion processes in the uterine epithelium represent a more complex system. Although considerable changes in lipid composition are indeed found, these do not fulfill predictions from model experiments in detail. Additional components (e.g., integral membrane proteins) may play a decisive role in this biological system.

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Edited by

Hans-Werner Denker

University Clinics
Essen, Federal Republic of Germany

and

John D. Aplin

St. Mary's Hospital
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